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(54) Title: UNIVERSAL CHEMISTRY ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF mRNA EXPRESSION

(57) Abstract

A kit for the quantitation of mRNA expression levels in a biological sample is provided which contains a detergent-chaotrope lysis mixture for extracting total RNA from the biological sample; a mixture of reverse transcriptase, nucleotides and an oligo-dT primer to synthesize cDNA from polyadenylated mRNA in the total mRNA; a means for labeling a primer or probe with a molecule which binds to an agent coated on a microtiter plate; a microtiter plate coated with an agent capable of binding to said molecule; a means for labeling a primer or probe with a detectable label; an antibody which detects the labeled primer or probe; and a means for detecting the antibody. Methods for quantitating mRNA expression in a biological sample are also provided.

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UNIVERSAL CHEMISTRY ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF mRNA EXPRESSION

BACKGROUND OF THE INVENTION

Polymerase chain reaction (PCR) has been recognized as a powerful technology for the characterization and quantitation of DNA. Recent methodologies for the direct performance of PCR on washed blood cells and whole blood significantly increase its usefulness for the processing and characterization of large numbers of blood samples. Wu et al. *Transgenica* **1994**, 1(1):35-38.

Analyses of PCR products using an enzyme-linked immunosorbent assay have been disclosed by several investigators. For example, Rasmussen et al., *Clinical Chemistry* **1994**, 40(2):200-205 teach detection of an immobilized, amplified product in a one phase system. This assay integrates the polymerase chain reaction with hybridization of the amplified product for detection in the same microwell. Detection of bovine leukemia virus and Salmonella was demonstrated, however, it is suggested that the assay is easily adaptable for other organisms, simply by using other primers and probes. However, because of poor reproducibility, this assay is recommended only for qualitative analysis. Genemed Biotechnologies, Inc. (South San Francisco, CA) also provides several ColorTect® enzyme-based signal generation kits which can be used to study several PCR samples simultaneously using a 96 microwell plate format.

The quantitation of RNA can also be performed by a similar technique, however, using RNA as the starting material

requires a reverse transcription step to synthesize a cDNA template for PCR. This method is referred to as RT-PCR. Quantitation of messenger RNA is achieved by RT-PCR.

A reverse transcription (RT) *in situ* PCR protocol in which direct incorporation of digoxigenin-11-dUTP allows the detection of PCR amplified RNA (via cDNA) in intact cells is described by Nuovo, G.J., *Biochemica* **1994**, 11(1):4-6. In this method, after digesting all non-target DNAs with RNase-free DNase, the desired cDNA is specifically amplified by first performing reverse transcription with a downstream primer and then *in situ* PCR with digoxigenin-11-dUTP. Incorporated digoxigenin-11-dUTP is then detected with alkaline phosphatase-conjugated antidigoxigenin and the colorimetric alkaline phosphatase substrates NBT and BCIP. This method of RT *in situ* PCR was compared to standard *in situ* hybridization and found to be more sensitive in the detection of hepatitis C virus. It is suggested that the detection of digoxigenin-labeled PCR-amplified viral RNAs and mRNAs can provide information not obtainable with either solution-phase PCR or standard *in situ* hybridization.

It has now been found that mRNA expression levels for various proteins can be quantitated in biological samples using a single assay which combines RT-PCR with ELISA. The determination of mRNA expression levels can be used in the early diagnosis of disease states and in monitoring the efficacy of potential therapeutics for these diseases.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a kit for the quantitation of mRNA expression levels in biological samples comprising a detergent-chaotrope lysis mixture for extracting total RNA from a biological sample; a mixture of reverse transcriptase, nucleotides and an oligo-dT primer to synthesize cDNA from the polyadenylated mRNA in the total mRNA population; a means for labeling a primer or probe with a molecule which binds to an agent coated on a microtiter plate; a means for labeling a primer or probe with a detectable label;

an antibody which detects the labeled primer or probe; a means for detecting the antibody; and a coated microtiter plate.

Another object of the present invention is to provide a method for quantitating levels of mRNA expression. This method comprises extracting total RNA from a biological sample. cDNA is then synthesized from mRNA in the extracted total RNA. Nucleotides, Taq polymerase, a magnesium source and a 5' and 3' primer selected in accordance with the mRNA to be quantitated, wherein the 5' primer is labeled with either a molecule capable of binding to a coated microtiter plate or a detectable marker, are then added to the cDNA and a PCR reaction is performed to obtain a PCR product. A probe is then added to the PCR product under conditions in which the probe hybridizes to the PCR product, said probe being capable of hybridizing to the PCR product and containing either (1) a molecule capable of binding to a coated microtiter plate, or (2) a detectable marker. The sample which contains a mixture of hybridized probe and PCR product, unhybridized probe and PCR products, and excess primers is then transferred to a microtiter plate coated with an agent capable of binding to the molecule on the PCR product or probe under conditions in which this molecule will bind to the microtiter plate. Detectable marker on bound hybridized probe and PCR product is then detected and mRNA levels determined.

DETAILED DESCRIPTION OF THE INVENTION

In many disease states it is faster, as well as more revealing, to track the mRNA expression levels of a selected protein for diagnosis of the disease as well as monitoring of therapies for the disease rather than wait for the production of the selected protein. In other cases, such as transplantation rejection, the event has occurred before the diagnosis can be made and a cytokine protein can be detected. The detection of expression levels of cytokine mRNA can precede this and be revealed while the rejection process can still be mitigated.

In the present invention, an assay is provided for the detection of mRNA expression of a selected protein in a biological sample using a universal enzyme-linked immunosorbent assay (ELISA) of probed polymerase chain reaction (PCR) products resulting from reverse transcriptase-PCR (RT-PCR) of the mRNA of interest. By "biological sample" it is meant to include, but is not limited to, whole blood, serum, plasma, urine, synovial fluid, saliva, cerebrospinal fluid, tissue infiltrate, cervical or vaginal exudate, pleural effusion, bronchioalveolar lavage fluid, gastric lavage fluid, small or large bowel contents, and swab specimens from various bodily orifices dispersed in a suitable medium. In a preferred embodiment, whole blood is used. In a preferred embodiment, detection of the mRNA is performed by well known colorimetric or fluorometric methods. The assay is constructed in a universal chemistry format wherein different RT-PCR primers and probes can be used for the detection of mRNA of a selected protein.

For example, in one embodiment, the present invention can be used to detect cellular levels of cytokine mRNA such as interleukin-8 (IL-8) messenger RNA in whole blood. PCR primers for cytokines such as β-actin, IL-1α, IL-1β, TNF-α, IL-6, IL-8 and GM-CSF which can be used in the present invention are described by Zhong et al., *Arch. Surg.* **1993**, 128:158-163.

In another embodiment, the present invention can be used to detect the cytokines, interleukin-2 (IL-2) and interferon-γ (IFN-γ). Elevated mRNA expression levels of these cytokines can be used to detect acute rejection of renal transplants prior to any other present method of diagnosis. Lipmann et al., *Transplantation* **1992**, 53:73-79. Primer and probe sequences which can be used for the detection of IL-2 include: the 5' primer 5'-CATTGCACTAAGTCTTGCACTTGTCA-3' (SEQ ID NO: 1); the 3' primer 5'-CGTTGATATTGCTGATTAAGTCCCTG-3' (SEQ ID NO: 2); and the probe 5'-TTCTTCTAGACACTGAAGATGTTTCAGTTC-3'(SEQ ID NO: 3). Primer and probe sequences which can be used for the detection of IFN-γ include: the 5' primer 5'-GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC-3' (SEQ ID NO: 4); the 3'

primer 5'-CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG-3' (SEQ ID NO: 5); and the probe 5'-TCTGGTCATCTTTAAAGTTTTTAAAAAGTT-3' (SEQ ID NO: 6). In a preferred embodiment, the 5' primer is labeled with biotin at the 5' end of the sequence and the probe is labeled with fluorescein isothiocyanate (FITC) at the 5' end of the sequence.

In yet another embodiment, the present invention can be used to detect placental isoferritin (PLF) messenger RNA. PLF mRNA expression levels have been related to the development of breast cancer. Breast cancer is difficult to diagnose at an early stage, when most amenable to therapy, and knowledge about the expression levels of PLF mRNA is believed to be a definitive way to diagnose and begin therapy as soon as possible. The efficacy of the therapy can also be monitored by following levels of mRNA PLF using the present invention.

In an assay performed in accordance with the present invention, total RNA is extracted from a biological sample. A mixture comprising reverse transcriptase, which is RNA-dependent DNA polymerase, nucleotides and an oligo-dT primer is then added to the extracted RNA to synthesize cDNA from the polyadenylated mRNA in the total mRNA population. In the same tube in which the cDNA is synthesized, more nucleotides, Taq polymerase, which is thermally stable DNA-dependent polymerase, a magnesium source and two primers selected in accordance with the mRNA to be detected are added and the PCR reaction run. The two primers are designed to prime inward from the ends of the known sequence of cDNA which represents the salient portion of the gene. In a first embodiment, the 5' primer is labeled with a molecule capable of binding to a coated microtiter plate. For example, the 5' primer can be labeled with biotin and the microtiter plate coated with streptavidin or the 5' primer can be labeled with digoxigenin and the microtiter plate coated with anti-digoxigenin antibody. In this embodiment, a probe designed to complement a selected portion of the target gene is then added to the tube. By "selected portion", it is meant that portion of the gene sequence which is not primed by the PCR primers. The probe is labeled with a detectable marker

such as FITC or digoxigenin and is capable of hybridizing to the labeled PCR product. In a second embodiment, the 5' primer is labeled with a detectable marker such as fluorescein isothiocyanate (FITC) or digoxigenin. In this embodiment, the probe, also designed to complement a selected portion of the target gene, is labeled with a molecule capable of binding to a coated microtiter plate. For example, the probe can be labeled with biotin and the microtiter plate coated with streptavidin or the probe can be labeled with digoxigenin and the microtiter plate coated with anti-digoxigenin antibody. The mixture of hybridized probe and PCR product, unhybridized probe and PCR products and excess primers are then added to a microtiter plate coated with an agent capable of binding to the molecule on either the PCR product or the probe. In a preferred embodiment, the microtiter plate is coated with streptavidin. A detectably labeled antibody capable of binding to any labeled PCR product which is hybridized to the probe and bound to the plate is then added and the antibody is detected. In a preferred embodiment, the antibody is detected colorimetrically or fluorometrically. For example, in one embodiment wherein the PCR product or probe is labeled with FITC, the antibody may comprise anti-FITC horse radish peroxidase which is detectable colorimetrically by the addition of 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate or O'phenylenediamine (OPD). In another embodiment wherein the PCR product or probe is labeled with FITC, the antibody may comprise anti-FITC alkaline phosphatase which is detectable fluorometrically by the addition of methylumbelliferyl phosphate (MUP).

In the present invention kits comprising components used in this universal enzyme-linked immunosorbent assay of probed PCR products resulting from reverse transcriptase-PCR (RT-PCR) of a particular mRNA of interest are also provided. Kits of the present invention are constructed using universal chemistry format in that the only differences are the RT-PCR primers and probe for the targets of interest. The remainder of the assay and kit components remain the same and can be routinely used

with various primers and probes for any target of interest. The construction of these kits in a universal chemistry manner provides significant advantage for the research and clinical setting.

Kits of the present invention comprise a detergent-chaotrope lysis mixture for extracting total RNA from a biological sample; a mixture of reverse transcriptase, nucleotides and an oligo-dT primer to synthesize cDNA from the polyadenylated mRNA in the total mRNA population; a means for labeling a primer or probe with a molecule which binds to an agent coated on a microtiter plate; a means for labeling a primer or probe with a detectable label, an antibody which detects the labeled primer or probe; a means for detecting the antibody; and a microtiter plate coated with an agent capable of binding to the molecule on either the probe of the PCR product. With this kit, the investigator need only provide the specific primers and probes to be used for a selected target to be measured.

The following nonlimiting examples are provided to further illustrate the present invention

EXAMPLES

Example 1: Isolation of RNA from whole blood

Whole blood (50 µl) was pelleted and washed twice with a NaCl-EDTA buffer in the following manner. The whole blood was mixed with 500 µl of 10 mM NaCl-EDTA buffer, vortexed vigorously and spun in a microcentrifuge at 14,000 for 5 minutes. The supernatants were poured off and the pellets washed a second time in the NaCl-EDTA buffer. Following a second centrifugation, the pellets were resuspended in 500 µl denaturation solution (5 M guanidinium thiocyanate, 0.125 M Tris-HCl (pH 7.4), 0.3125 M sodium acetate, and 1.25% β-mercaptoethanol), 500 µL TRAx® lysis buffer (1.56 dibasic potassium phosphate, 0.14 g monobasic potassium phosphate, 8.77 g sodium chloride, 90 ml Triton-X and 60 ml Nonidet P-40) and 100 µg tRNA. The lysis mixture was vigorously vortexed and the tubes incubated at 65°C for 10 minutes. The tubes were iced

for at least 1 minute, vortexed, and then split into a fresh tube so that 500 µl isopropanol could be added to each tube. The tubes were vortexed at high speed for 10 seconds each, then centrifuged at 14,000 RPM for 15 minutes. The supernatants were poured off, and the visible white pellets washed with 1.5 ml 70% ethanol and centrifuged at 14,000 RPM for 10 minutes. The supernatants were poured off and the pellets dried in a Speed-Vac with no heat for one hour. The dried pellets were resuspended in 50 µl sterile water and frozen at -70°C. Before use, the pellets were heated at 50°C-65°C until they dissolved in water.

Example 2: Synthesis of cDNA

A mixture of reverse transcriptase, which is RNA-dependent DNA polymerase, nucleotides and an oligo-DT primer are added to synthesize cDNA from the polyadenylated mRNA in the isolated total RNA population. This procedure requires temperature which are achieved in the same thermal cycler as described in Example 3.

Example 3: PCR reaction

In the same tube in which the cDNA is synthesized, additional nucleotides, Taq polymerase, a source of magnesium and the two primers are added and PCR reaction run in the thermal cycler for 35 cycles. The primers are designed to prime inwardly from the ends of the known sequence of cDNA which represents the salient part of the gene. The 5' primer is biotin-labeled and the 3' primer is unlabelled. The resultant biotin-labelled PCR products are hybridized to the fluorescein isothiocyanate (FITC)-labelled probed in the same tube. The probe is designed to complement a selected portion of the target sequence which is not primed by the PCR primers.

This PCR reaction is asymmetrical yielding a single-stranded PCR product. This reaction is accomplished by using an excess amount of biotin-labelled 5' primer (50 µM) and a limiting amount of the unlabelled 3' primer (1 µM). The

resultant single-stranded, biotin-labelled PCR product then easily hybridizes with a FITC labeled probe.

Example 4: ELISA assay

The mixture of hybridized FITC-labelled probe and biotin-labelled PCR product, unhybridized probes and PCR products and excess primers are added to a microtiter plate coated with streptavidin. All versions of the biotin-labelled primer or PCR product bind to the streptavidin quickly and very strongly during a 30 minute room temperature incubation. Subsequent wash steps remove all unhybridized probes and unlabeled primers. The FITC-labeled probes hybridize only to the full length biotin-labeled PCR products, and only those which are hybridized to the bound-labelled PCR products remain in the wells after the washes.

The bound hybrid, consisting of FITC-labelled probe and the biotin-labelled PCR product, bind sheep anti-FITC horseradish peroxidase (HRP) conjugated during incubation at room temperature. After another wash step, the TMB-peroxidase substrate (3,3', 5,5'-tetramethylbenzidine) is added and the color is allowed to develop. The reaction is stopped by the addition of a weak acid solution. The plate is then read in an ELISA plate reader.

Example 5: Determining IL-8 mRNA Expression

mRNA was isolated from whole blood as described in Example 1. The RNA pellet was resuspended in 20 μl of RNase-free water. The RNA suspension (10 μl) plus oligo dT primer (1 μl; 0.5 μg/μl) and RNase-free water (2 μl) were incubated at 70°C for 10 minutes. The samples were then placed on ice for 1 minute and 7 μl of master mix containing 10X PCR buffer, 25 mM $MgCl_2$, 10 mM each dNTP mix and 0.1 M DTT was added. The samples were incubated at 42°C for 5 minutes. Reverse transcriptase (1 μl) was added and the samples were incubated again at 42°C for 50 minutes, followed by a 15 minute incubation at 70°C. The samples were then chilled on ice.

The asymmetric PCR reaction was then performed using 10X PCR buffer, 25 mM $MgCl_2$, 10 mM each dNTP, Taq polymerase (2 units/reaction), 50 μM of human IL-8 5′ primer: 5′-biotin-ATGACTTCCAAGCTGGCCGTGGCT-3′ (SEQ ID NO: 7), and 1 μM human IL-8 3′ primer: 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′ (SEQ ID NO: 8) for 30 to 35 cycles.

Thirty microliters of the resulting PCR product were then hybridized with 20 μl of a hybridization mixture containing 100 mM HEPES, 0.1% Tween 20, 200 μM EDTA, 1 M NaCl and 20 pmol of a human IL-8 probe: 5′-FITC-CACAGAGCTGCAGAAATCAGGAAGGCTGCCA-3′ (SEQ ID NO: 9). The mixture was then incubated at 95°C for 5 minutes followed by a 1.5 hour incubation at 37°C. An ELISA assay was performed in the hybridized mixture as described in Example 4.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Constance Emmett, Kimberly A. Foster

(ii) TITLE OF INVENTION: Universal Chemistry Enzyme-Linked Immunosorbent Assay for Detection of mRNA Expression

(iii) NUMBER OF SEQUENCES: 9

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(v) COMPUTER READABLE FORM:

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(C) REFERENCE/DOCKET NUMBER: TCEL-0039

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CATTGCACTA AGTCTTGCAC TTGTCA 26

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CGTTGATATT GCTGATTAAG TCCCTG 26

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTCTTCTAGA CACTGAAGAT GTTTCAGTTC 30

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCATCGTTTT GGGTTCTCTT GGCTGTTACT GC 32

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTCCTTTTTC GCTTCCCTGT TTTAGCTGCT GG 32

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCTGGTCATC TTTAAAGTTT TTAAAAAGTT 30

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATGACTTCCA AGCTGGCCGT GGCT 24

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TCTCAGCCCT CTTCAAAAAC TTCTC 25

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CACAGAGCTG CAGAAATCAG GAAGGCTGCC A 31

What is claimed is:

1. A kit for the quantitation of mRNA expression levels in a biological sample comprising:

a) a detergent-chaotrope lysis mixture for extracting total RNA from a biological sample;

b) a mixture of reverse transcriptase, nucleotides and an oligo-dT primer to synthesize cDNA from polyadenylated mRNA in the total mRNA;

c) a means for labeling a primer or probe with a molecule which binds to an agent coated on a microtiter plate;

d) a microtiter plate coated with an agent capable of binding to said molecule;

e) a means for labeling a primer or probe with a detectable label;

f) an antibody which detects the labeled primer or probe; and

g) a means for detecting the antibody.

2. A method for quantitating levels of mRNA expression comprising:

a) extracting total RNA from a biological sample;

b) synthesizing cDNA from mRNA in the extracted total RNA;

c) adding nucleotides, Taq polymerase, a magnesium source and a 5′ and 3′ primer selected in accordance with the mRNA to be detected to the cDNA, wherein said 5′ primer is labeled with a molecule capable of binding to a coated microtiter plate;

d) performing a PCR reaction to obtain a PCR product;

e) adding a probe to the PCR product which is capable of hybridizing to the PCR product and labeled with a detectable marker under conditions in which the probe hybridizes to the PCR product;

f) transferring a mixture of hybridized probe and PCR product, unhybridized probe and PCR products and excess primers to a microtiter plate coated with an agent capable of

binding to the molecule on the PCR product under conditions in which the molecule on the PCR product will bind to the microtiter plate; and

g) detecting the detectable marker on bound hybridized probe and PCR product so that mRNA levels can be determined.

3. A method for quantitating levels of mRNA expression comprising:

a) extracting total RNA from a biological sample;

b) synthesizing cDNA from mRNA in the extracted total RNA;

c) adding nucleotides, Taq polymerase, a magnesium source and a 5' and 3' primer selected in accordance with the mRNA to be detected to the cDNA, wherein said 5' primer is labeled with a detectable marker;

d) performing a PCR reaction to obtain a PCR product;

e) adding to the PCR product a probe capable of hybridizing to the PCR product and containing a molecule capable of binding to an agent coated on a microtiter plate under conditions in which the probe will bind to the PCR product;

f) transferring a mixture of hybridized probe and PCR product, unhybridized probe and PCR products and excess primers to a microtiter plate coated with an agent capable of binding to the molecule on the probe under conditions in which the molecule on the probe will bind to the microtiter plate; and

g) detecting the detectable marker on bound hybridized probe and PCR product so that mRNA levels can be determined.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12Q 1/68; C12N 15/00; C07H 21/04
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 172.3, 810; 436/501; 514/44; 536/24,3; 935/77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MANIATIS et al., "Molecular Cloning, A laboratory manual". New York: Cold Spring Harbor Laboratory, 1982, page 212-216 and 280-281, see entire document.	1-3
Y	MATTHEWS et al. Review, Analytical Strategies for Use of DNA Probes. Analytical Biochemistry, 1988, Volume 169, pages 1-25, especially page 1 and Figure 10 page 17.	1-3
Y	SYVANEN et al. Fast quantification of nucleic acid hybrids by affinity-based hybrid collection. Nucleic Acids Research, 1986, Volume 14, Number 12, pages 5037-5048. See whole document.	1-3

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search 21 JUNE 1996	Date of mailing of the international search report 12 JUL 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEZIA RILEY Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08142

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SYVANEN et al. Quantification of polymerase chain reaction products by affinty-based hybrid collection. Nucleic Acids Research, 1988, Volume 16, Number 23, pages 11327-11338. See whole document.	1-3

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08142

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN

search terms: Nucleic, PCR or Polymerase chain reaction, quantitative, matrix, solid support